RECOMBINANT GENES FOR POLYKETIDE MODIFYING ENZYMES

Cross-Reference To Related Applications

[0001] This application claims benefit of U.S. Provisional Patent Application No. 60/393,016, filed June 28, 2002, which is incorporated herein by reference in its entirety.

Field of the Invention

[0002] The present invention provides methods and materials for modifying polyketides by the addition of carbohydrate and other moieties to the polyketides. Polyketides are a diverse class of compounds with a wide variety of activities, including activities useful for medical, veterinary, and agricultural purposes. The present invention therefore relates to the fields of molecular biology, chemistry, recombinant DNA technology, medicine, animal health, and agriculture.

Background of the Invention

[0003] Modular PKS enzymes are large, multi-subunit enzyme complexes that perform the biosynthesis of polyketide secondary metabolites. See O'Hagan, D., 1991 (a full citation of any reference referred to herein by last name of first author and year of publication is located at the end of this section). Examples of polyketides made by modular PKS enzymes include the antibiotic erythromycin, the immunosuppressant FK506, and the antitumor compound epothilone. See also PCT patent publication No. 93/13663 (erythromycin); U.S. Patent No. 6,303,342 B1 (epothilone); U.S. Patent No. 6,251,636 B1 (oleandolide); PCT publication WO 01/27284 A2 (megalomicin); U.S. Patent No. 5,098,837 (tylosin); U.S. Patent No. 5,272,474 (avermectin); U.S. Patent No. 5,744,350 (triol polyketide); and European patent publication No. 791,656, now U.S. Patent No. 5,945,320 (platenolide), each of which is incorporated herein by reference.

[0004] PCT publication WO 01/27284 A2 referenced above discloses the desosamine biosynthesis gene *megCII* encoding a 3,4-isomerase and glycosylyltransferase gene *megCIII*; the mycarose biosynthesis genes *megBII* (*megBII-2*)and *megBIV* encoding a 2,3-reductase and 4-ketoreductase respectively, and the mycarose glycosyltransferase gene *megBV*; the megosamine biosynthesis genes *megDII*, *megDIII*, *megDIV*, *megDV*, and *megDVI*, and the megosamine glycosyltransferase gene *megDI*. That publication made partial disclosures of *megBVI* (*megT*)

and *megF*. The *megBVI* gene, which has dual function in mycarose and megosamine biosynthesis as a 2,3-dehydratase, was only partially disclosed (less than 10% of the nucleotide sequence) and was named *megT*. The *megF* genes sequence was disclosed in part (47%).

[0005] A large interest in PKS enzymes arises from the ability to manipulate the specificity or sequence of reactions catalyzed by PKSs to produce novel useful compounds. See U.S. Patent 5,962,290 and McDaniel, R., *et al.*, 2000, and Weissman, K.J *et al.* 2001. A number of plasmid-based heterologous expression systems have been developed for the engineering and expression of PKSs, including multiple –plasmid systems for combinatorial biosynthesis. See McDaniel, *et al.*, 1993, Xue, *et al.*, 1999, and Ziermann, *et al.*, 2000, and U.S. Patent Nos. 6,033,883 and 6,177,262; and PCT publication Nos. 00/63361 and 00/24907, each of which is incorporated herein by reference. Polyketides are often modified by P450 enzymes that hydroxylate the polyketide and by glycosyl transferase enzymes that glycosylate the polypeptide. Using recombinant technology, see PCT Pub. No. 98/49315, incorporated herein by reference, one can also hydroxylate and or glycosylate polyketides. Such technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters.

[0006] The class of polyketides includes the megalomicins, which are 6-*O*-glycosides of erythromycin C with acetyl or propionyl groups esterified to the 3''' or 4''' hydroxyls of the mycarose sugar. They were reported in 1969 as antibacterial agents produced by *Micromonospora megalomicea* sp. n. (Weinstein *et al.*, 1969). The deoxyamino sugar at C-6 was named 'megosamine" (Nakagawa *et al.*, 1984). Therapeutic interest in megalomicin arose from several observed biological activities, including anti-bacterial activity, effects on protein trafficking in eukaryotic cells, inhibition of vesicular transport between the medial and *trans* Golgi, resulting in undersialylation of proteins, inhibition of the ATP-dependent acidification of lysosomes, anomalous glycosylation of viral proteins, antiviral activity against herpes, and as potent antiparasitic agents. Megalomicins are effective against *Plasmodium falciparum, Trypanosoma* sp. and *Leishmania donovani* (Bonay *et al.*, 1998). As erythromycin does not have antiparasitic activity, the antiparasitic action of megalomicin is most probably related to the presence of the megosamine deoxyamino sugar at C-6.

[0007] The aglycone backbone of both megalomicin and erythromycin is the complex polyketide 6-deoxyerythronolide B (6-dEB), produced from the successive condensations of a propionyl-CoA starter unit and 6 methylmalonyl-CoA extender units (Figure 2). Complex polyketides are assembled by modular polyketide synthases (PKSs), which are composed of multifunctional polypeptides that contain the activities (as enzymatic domains) for the condensation and subsequent reductions required to produce the polyketide chain (Katz, 1997; Cane *et al.,* 1998).

[0008] The biosynthetic pathway of megalomicin is shown in Figure 2. Both the megalomicin and erythromycin pathways are identical through the formation of erythromycin C, the penultimate intermediate of erythromycin A and megalomicin A. The megalomicin biosynthetic gene cluster has, in addition to the genes for the synthesis and attachment of the mycarose and desosamine sugars, a set of genes for synthesis and attachment of the unique deoxysugar L-megosamine. Making glycosylated and or/hydroxylated derivatives of aglycones through genetic engineering would be possible if one could transfer one or more of the megalomicin sugar biosynthesis and glycosyl-transferase, and P450 monooxygenase genes to another host. There exists a need for methods and materials to modify polyketides by P450 modification and/or the addition of sugar moieties to create active compounds in heterologous or native hosts. The present invention provides methods and compositions to meet those and other needs.

[0009] The following articles provide background information relating to the invention and are incorporated herein by reference.

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Summary of the Invention

[0010] As described above, portions of the megalomicin PKS gene cluster DNA sequence have been disclosed in PCT publication WO 01/27284 A2. That publication disclosed the DNA sequence of mycarose biosynthesis genes BII (BII-2) and BIV and mycarose transferase gene *megBV*, desosamine biosynthesis gene *megCII* and desosamine transferase gene *megCIII*, and megosamine biosynthesis genes *megDII*, *megDIII*, *megDIV*, *megDV*, and *megDVI*, *megDVII* and megosamine transferase gene *megDI*, as well as a partial DNA sequence of *megBVI* (*megT*), which has dual function in mycarose and megosamine biosynthesis pathways, and *megF*.

[0011] The present invention provides the complete nucleotide sequence of the *megF* and *megK* genes, which encode monooxygenases of P450-type that hydroxylate at the C-6 and C-12 position of 6-dEB as well as recombinant vectors and host cells comprising such genes. The present invention also provides recombinant vectors and host cells comprising the genes *megBIII* and/or *megBVI* of the mycarose biosynthesis pathway (*megBVI* also functions in the megosamine biosynthesis pathway as a 2,3-dehydratase), *megCIV* and *megCV* of the desosamine biosynthesis pathway, and *megBVI* (formerly designated *megT*) of the megosamine biosynthesis pathway. The present invention also provides novel genes in recombinant form common to several desoxysugar biosynthesis pathways, including *megM* encoding a megosamine 6-dehydrogenase, and *megL* encoding a TDP-glucose synthase. The present invention also provides a recombinant PKS cluster regulatory gene *megR* isolated from the upstream region of the megalomicin PKS cluster. The recombinant genes of the present invention may be isolated from *Micromonospora megalomicea*, sp. nigra.

[0012] The present invention provides recombinant methods and materials for expressing genes useful in P450-mediated oxidation of a polyketide and/or the biosynthesis and transfer to a polyketide of mycarose, desosamine, and/or megosamine in recombinant host cells. More specifically, the genes and proteins isolated from *Micromonospora megalomicea*, sp. nigra, of the present invention are useful in the hydroxylation and glycosylation of polyketides by the addition of mycarose, desosamine, and/or megosamine to a polyketide. In particular the invention provides recombinant monooxygenases of P450 type *megK* and *megF*; recombinant mycarose synthesis genes *megBIV*, *megBII (meg BII-2)*, *megBIII*, *megBVI*, and *megDIV* and recombinant mycarose transfer gene *megBV*; recombinant desosamine synthesis genes *megCII*,

megCIV, *megCV*, *meg DII*, *meg DIII* and recombinant *megCIII* desosamine transfer gene; recombinant megosamine synthesis genes *megDII*, *megDIII*, *megDIV*, *megDV*, *megDVII*, *megDVI*, *megBVI* and the megosamine transfer gene *megDI*; and recombinant deoxysugar genes *megM* encoding a glucose-6-dehydratase, and *megL* encoding a TDP-glucose synthase (common to the desosamine, mycarose, and megosamine biosynthesis pathways). The invention also provides the proteins encoded by the recombinant genes of the present invention in isolated, purified, and/or recombinant form. The invention also provides novel polyketides produced by glycosylation mediated by the sugar biosynthesis and transfer genes and/or by hydroxylation mediated by the P450 genes isolated from the megalomicin PKS gene cluster of *Micromonospora megalomicea*, sp. nigra.

[0013] Thus, in one embodiment, the invention provides recombinant DNA compounds that comprise the C-6 hydroxylase (the *megF* gene), and C-12 hydroxylase (the *megK* gene), the desosamine biosynthesis and desosaminyl transferase enzymes and the recombinant proteins that can be produced from these nucleic acids in the recombinant host cells of the invention. In some embodiments, the invention provides an isolated, purified, or recombinant nucleic acid comprising a polyketide modifying gene, wherein said gene encodes one of the polyketide modifying enzymes MegR, MegF, MegK, MegCIV, MegCV, MegBVI, MegBIII, MegL, or MegM. In some embodiments, the nucleic acid is less than about 9.0 kilobases in length. In some embodiments, the nucleic acid does not also comprise one or more of the polyketide modifying genes *megBI, megBV, megBIV, megCI, megCII, megDII, megDIII, megDIV, megDV, megDVII,* and *megY*. In some embodiments, the gene encodes one of the polyketide modifying enzymes MegR, MegK, MegCIV, MegCV, or MegBVI. In some embodiments, the gene encodes one of the polyketide modifying enzymes MegF, MegBIII, MegL, or MegM. In some embodiments, the invention provides an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of mycarose to a polyketide, where the genes include the *megM, megL, megBIII, megBIV, megDIV, meg BV, meg BII (megBII-2),* and *meg BVI* genes , and, optionally, the *megF* gene. In some embodiments, the polyketide modifying enzyme has an amino acid sequence that is encoded by SEQ ID NO: 1 or SEQ ID NO: 2., or hybridizes to SEQ ID NO: 1 or SEQ ID NO: 2 under stringent conditions, or has at least about 90% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the polyketide modifying gene is operably linked to a heterologous promoter. In some

embodiments, the invention provides an isolated, purified, or recombinant nucleic acid that contains a polyketide modifying enzyme gene *meg*K, *meg*CV, *meg*CIV, *meg*R, *meg*BVI, *meg*F, *meg*BIII, *meg*L, or *meg*M.

[0014] The invention further provides isolated, purified, or recombinant nucleic acids containing genes for the biosynthesis and attachment of glycosyl units to a polyketide. In one embodiment, the invention provides isolated, purified, or recombinant nucleic acids containing genes for the biosynthesis and attachment of mycarose to a polyketide and/or hydroxylation of the polyketide, where the genes include the genes that encode the enzymes MegM, MegL, MegBIII, MegBIV, MegDIV, MEG BII (MegBII-2), Meg BVI, optionally MegBV, and, optionally, MegF. In another embodiment, the invention provides an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of megosamine to a polyketide, where the genes may include the genes that encode the enzymes MegM, MegL, MegCII, MegBVI, MegDIV, MegDV, MegDII, and MegDIII enzymes, and, optionally the MegDI enzyme. In a further embodiment, the invention provides an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of megosamine to a polyketide, where the genes may include the genes that encode the enzymes MegM, MegL, MegCII, MegBVI, MegDIV, MegDVI, MegDVII, MegDII, and MegDIII enzymes, and, optionally, the MegDI enzyme. In yet a further embodiment, the invention provides an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of desosamine to a polyketide, where the genes include the genes that encode the enzymes MegM, MegL, MegCII, MegCIV, MegCV, MegDII, and MegDIII enzymes, and, optionally, the MegCIII enzyme.

[0015] The invention also provides materials that include recombinant DNA compounds that encode the PKS modification enzymes TDP-hexose synthase (the *megL* gene for attachment of thymidinediphospho(TDP) glucose), and TDP hexose-4,6-dehydratase (the *megM* gene), and the recombinant proteins that can be produced from these nucleic acids in the recombinant host cells of the invention.

[0016] The invention also provides materials that include recombinant DNA compounds that encode the PKS cluster regulatory gene (*megR*).

[0017] The invention also provides a vector comprising the modifying genes *megCII*, *megCIII*, *megBII*, *megK*, *megF*, *megBIII*, *megM*, and *megL*.

[0018] The invention also provides a vector comprising the modifying genes *megK*, *megCV*, *megCIV*, and *megBVI*.

[0019] The invention also provides expression vectors that contain at least one of the polyketide modifying genes described above, e.g., a vector where the gene is operably linked to a promoter. In some embodiments, the polyketide modifying gene is *megR*, *megF*, *megK*, *megCIV*, *megCV*, *megBVI*, *megBIII*, *megL*, or *megM*.

[0020] The invention further provides cosmid vectors that contain at least one of the polyketide modifying genes described above.

[0021] The invention further provides recombinant host cells containing at least one of the polyketide modifying genes described above. In some embodiments, the host cell expresses a polyketide modifying enzyme, where the enzyme is the MegK or MegF monooxygenase. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a desosamine biosynthetic gene set, where the enzyme is MegCIV, MegCV, or MegCIII. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a desosamine biosynthetic gene set, where the enzyme is MegCII, MegCIV, MegCV, or MegCIII. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a megosamine biosynthetic gene set, where the enzyme is MegBVI or MegDI. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a megosamine biosynthetic gene set, where the enzyme is MegDI, MegDII, MegDIII, MegDIV, MegDV, MegDVI, MegDVII, or MegBVI. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a mycarose biosynthetic gene set, where the enzyme is MegBIII or MegBVI. In some embodiments, the host cell expresses a polyketide modifying enzyme encoded by a gene from a mycarose biosynthetic gene set, where the enzyme is MegBII, MegBIII, MegBIV, MegBV, or MegBVI. The invention further provides host cells that expreess a polyketide modifying gene that encodes a polyketide modifying enzyme MegR, MegF, MegK, MegCIV, MegCV, MegBVI, MegBIII, MegL, or MegM.

[0022] The invention also provides methods using the recombinant genes of the present invention to modify aglycones or polyketides.

[0023] The invention also provides materials that include recombinant DNA compounds that encode the PKS modification enzymes effectuating mycarose biosynthesis and

glycosyltransferase enzymes and the recombinant proteins that can be produced from these nucleic acids in the recombinant host cells of the invention.

[0024] The invention also provides materials that include recombinant DNA compounds that encode the PKS modification enzymes effectuating desosamine biosynthesis and glycosyltransferase enzymes and the recombinant proteins that can be produced from these nucleic acids in the recombinant host cells of the invention.

[0025] The invention also provides materials that include recombinant DNA compounds that encode the PKS modification enzymes effectuating megosamine biosynthesis and glycosyltransferase enzymes and the recombinant proteins that can be produced from these nucleic acids in the recombinant host cells of the invention.

[0026] In one embodiment, the invention provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and/or purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) and/or recombinant (i.e., nucleic acid synthesized or otherwise manipulated *in vitro*) form. The DNA molecules of the invention may in some embodiments also comprise, in addition to sequences that encode polyketide modifying enzymes, sequences that encode polyketide synthase domains. For example, the DNA molecules of the invention may contain one or more sequences that encode one or more domains (or fragments of such domains) of one or more modules in one or more of the ORFs of the megalomicin or other PKS. Examples of PKS domains include the KS (beta-ketoacylsynthase), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), enoylreductase (ER), acyl carrier protein (ACP), and thioesterase (TE) domains, for example, domains of at least 6 extender modules and loading module of the three proteins encoded by the three ORFs of the megalomicin PKS gene cluster.

[0027] In one embodiment, the present invention provides recombinant PKS modification enzymes including those that synthesize mycarose, desosamine, and megosamine moieties, those that transfer those sugar moieties to the polyketide 6-dEB, and those that hydroxylate 6-dEB at C-6 or C-12 position.

[0028] In one embodiment, the invention provides a recombinant expression vector that comprises the desosamine biosynthetic genes and optionally a desosaminyl transferase gene. In a related embodiment, the invention provides recombinant host cells comprising the vector that

produces the desosamine biosynthetic gene products and optionally a desosaminyl transferase gene product. In a preferred embodiment, the host cell is *Streptomyces lividans* or *Streptomyces coelicolor*. The desosaminyl transferase gene and gene product may be from the megalomicin gene cluster or may be from a different gene cluster, for example, the desosaminyl transferase gene and gene product from the pikromycin or narbomycin gene clusters as described in U.S. Patent Nos. 6,509,455 and 6,303,767.

[0029] In one embodiment, the invention provides one or more recombinant expression vectors that comprise the desosamine and mycarose biosynthetic genes and, optionally, the desosaminyl and/or mycarosyl transferase genes. In a related embodiment, the invention provides recombinant host cells comprising the vector(s) that produces the desosamine and mycarosyl biosynthetic gene products and desosaminyl and mycarosyl transferase gene products. In a preferred embodiment, the host cell is *S. lividans* or *S. coelicolor*. As described above, the desosaminyl transferase gene and gene product and mycarosyl transferase gene and gene product may be from the megalomicin cluster or may be from a different gene cluster.

[0030] In one embodiment, the invention provides one or more recombinant expression vectors that comprise the desosamine, megosamine, and mycarose biosynthetic genes, and, optionally, a desosaminyl transferase, mycarosyl transferase, and/or megosamine transferase genes. In a related embodiment, the invention provides recombinant host cells comprising the vector(s) that produces the desosamine, megosamine and mycarosyl biosynthetic gene products and, optionally, desosaminyl, mycarosyl, and megosaminyl transferase gene products. In a preferred embodiment, the host cell is *S. lividans* or *S. coelicolor*. As described above, the desosaminyl transferase gene and gene product and mycarosyl transferase gene and gene product may be from the megalomicin cluster or may be from a different gene cluster.

[0031] In one aspect, the invention provides methods of producing a modified polyketide. In some embodiments, the method includes culturing a recombinant cell containing a nucleic acid of the invention under conditions in which the cell expresses a product of a gene encoded by the nucleic acid, and under conditions in which the unmodified polyketide is present, thereby producing the modified polyketide. In some of these embodiments the cell further contains a recombinant nucleic acid encoding at least one module of a polyketide synthase. In some embodiments, the cell produces megosamine and can attach megosamine to a polyketide, where the cell in its naturally occurring non-recombinant state cannot produce megosamine. In

one embodiment, the invention provides a method for desosaminylating polyketide compounds in recombinant host cells, which method comprises expressing the PKS for the polyketide and a desosaminyl transferase and desosamine biosynthetic genes in said host cells. In one embodiment, the invention provides a method for desosaminylating and mycarosylating polyketide compounds in recombinant host cells, which method comprises expressing the PKS for the polyketide and a desosaminyl and mycarosyl transferase and desosamine and mycarose biosynthetic genes in said host cells. In one embodiment, the invention provides a method for mycarosylating desosaminylating, and megosaminylating polyketide compounds in recombinant host cells, which method comprises expressing the PKS for the polyketide and a desosaminyl, megosaminyl, and mycarosyl transferase and desosamine, megosamine, and mycarose biosynthetic genes in said host cells.

[0032] In one embodiment, the host cell expresses a beta-glucosidase gene as well, and this method may be advantageous when producing desosaminylated polyketides in *Streptomyces* or other host cells, that glucosylate desosaminylated polyketides, which can decrease antibiotic activity. By coexpression of beta-glucosidase, the glucose residue is removed from the polyketide.

[0033] In one embodiment, the invention provides the *meg*K hydroxylase gene in recombinant form and methods for hydroxylating polyketides with the recombinant gene product. The invention also provides polyketides thus produced and the antibiotics or other useful compounds derived therefrom.

[0034] In one embodiment, the invention provides the *meg*CIV 4,5-dehydratase, *meg*CV reductase, *meg*BVI 2,3-dehydratase (also known as *meg*T) genes in recombinant form and methods for modifying polyketides with the recombinant gene product. The invention also provides polyketides thus produced and the antibiotics or other useful compounds derived therefrom.

[0035] The invention also provides novel polyketides or other useful compounds derived therefrom. The compounds of the invention can be used in the manufacture of another compound. In a preferred embodiment, the compounds of the invention are antibiotics formulated in a mixture or solution for administration to an animal or human.

[0036] These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

Brief Description of the Drawings

[0037] Figure 1 is a schematic of the megalomicin polyketide synthase (meg DEBS) and corresponding *meg* genes upstream and downstream of the meg DEBS region and cosmids overlapping this region.

[0038] Figure 2 is a schematic of the megalomicin biosynthetic pathway.

[0039] Figure 3 is a schematic of the biosynthetic pathways of the deoxysugars megosamine, mycarose, and desosamine in megalomicin synthesis.

Detailed Description of the Invention

(1) Introduction

[0040] The present invention provides novel genes of the megalomicin cluster in isolated, purified, and/or recombinant form, including genes of the mycarosyl biosynthesis pathway and transferase, desosamine biosynthesis pathway and transferase, megosamine biosynthesis pathway and transferase, the *megM* and *megL* genes common to deoxysugar synthesis, as well as the monooxygenases of P450 type MegK and MegF.

[0041] The present invention provides in isolated, purified, and/or recombinant form desosamine biosynthesis genes *megCII*, *megCIV*, *megCV*, *meg DII, meg DIII*, and the *megCIII* transferase gene, as well as the proteins encoded by those genes.

[0042] The present invention provides in recombinant form mycarose biosynthesis genes *megBIV*, *megBII(megBII-2)*, *megBIII, megBVI*, *megDIV*, and the *megBV* transferase gene, as well as the proteins encoded by those genes.

[0043] The present invention provides in isolated, purified, and/or recombinant form megosamine biosynthesis genes *megDII*, *megDIII*, *megDIV*, *megDV*, *megDVII*, *megDVI*, *megBVI (megT)*, and the *megDI* transferase gene, as well as the proteins encoded by those genes.

[0044] The present invention provides isolated, purified, and/or recombinant P450-like monooxygenase enzymes MegK and MegF, and the genes *megK* and *megF* in recombinant form.

[0045] The present invention provides isolated, purified, and/or recombinant deoxysugar genes *megM* encoding a meg glucose-6-dehydratase, and *megL* encoding a meg TDP-glucose synthase.

[0046] The present invention provides isolated, purified, and/or recombinant megalomicin cluster PKS regulatory gene *megR* and its control binding sequences, and protein encoded by its coding sequence.

[0047] The present invention further provides vectors containing the genes of the invention, as well as host cells containing the genes of the invention. The invention also provides methods of producing modified polyketides by culturing recombinant cells that contain the genes of the invention under conditions where one or more of the genes are expressed and the unmodified polyketide is present; in some cases the cell further contains a recombinant nucleic acid encoding at least one module of a polyketide synthase.

[0048] The invention further provides polyketides produced using the above nucleic acids and methods.

(2) Definitions

[0049] The present invention may be better understood with reference to the following definitions. Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

[0050] As used herein, 'nucleic acid' and 'polynucleotide' have their ordinary meanings and are used interchangeably. It will be appreciated that reference to one strand of a double-stranded molecule is intended to refer as well to the complementary strand, the sequence of which will be apparent to the practitioner. Exemplary nucleic acids are RNA and DNA; the latter is also referred to herein as 'DNA compounds.'

[0051] As used herein, 'recombinant' has its ordinary meaning in the art and refers to a nucleic acid synthesized or otherwise manipulated *in vitro* (e.g., 'recombinant nucleic acid'), to methods of using recombinant nucleic acids to produce gene products in cells or other biological systems, to a polypeptide (e.g., 'recombinant protein') encoded by a recombinant nucleic acids, or to cells comprising a recombinant nucleic acid (including progeny of cells into which a recombinant nucleic acid has been introduced).

[0052] As used herein, 'gene' refers to a nucleic acid sequence that encodes a useful product. A gene can encode an mRNA that is transcribed from the gene and translated by a ribosome into a protein. 'Extra copies' of a gene, e.g., 'extra copies of an *eryG* gene,' refers to a gene that is introduced into a cell that already contains a copy of the gene.

[0053] As used herein, 'polyketide modifying gene' or 'polyketide synthase (PKS) modifying gene' (used interchangeably herein) refers to a gene encoding a protein that effectuates glycosylation of an aglycone, including the biosynthesis of the glycosyl unit or sugar, or hydroxylation of an aglycone, to produce a 'modified polyketide,' i.e., a polyketide that has been modified from an aglycone and/or that has been modified by the addition of hydroxyls beyond those present in the polyketide as synthesized by the PKS core enzymes. Non-limiting examples of polyketide modifying genes and the proteins encoded by them are the *megF* gene (encoding a C-6 hydroxylase), the *megK* gene (encoding a C-12 hydroxylase); *megDI, megDII, megDIII, megDIV, megDV, megDVI, megDVII,* and *megBVI* genes (encoding enzymes of the megosamine biosynthetic pathway); *megCII, megCIV, megCV,* and *megCIII* (encoding enzymes of the desosamine biosynthetic pathway); and *megBII (megBII-2), megBIII, megBIV, megBV,* and *megBVI* (encoding enzymes of the mycarose biosynthetic pathway; *megR* (encoding a regulatory gene); *megL* (encoding a TDP-glucose synthase gene), and *megM* (encoding a hexose dehydratase). These are merely examples; other polyketide modifying genes are apparent from context and are described below. Enzymes and other regulatory proteins encoded by polyketide modifying genes are referred to herein as "polyketide modifying enzymes."

[0054] As used herein, 'heterologous' in reference to a polyketide modifying gene or protein in a recombinantly modified cell means a gene or protein not found in an unmodified cell of the same species or strain (e.g., a non-recombinant cell). One example of a heterologous gene is a gene from a first species that is introduced into a cell of a second species (e.g., by introduction of a recombinant polynucleotide encoding the gene). Another example of a heterologous gene is a gene (in a cell) that encodes a chimeric PKS.

[0055] As used herein, a promoter operably linked to a protein encoding sequence (gene) is 'heterologous' if it is not usually associated with the gene. In one embodiment a heterologous promoter is derived from a different species than the protein encoding sequence (for example a viral promoter that controls expression a bacterial gene). In another embodiment, a heterologous

promoter is from the same species but is not normally (i.e., in non-recombinant organisms) associated with the gene. A heterologous promoter may also be a synthetic promoter.

[0056] As used herein, 'host cell' refers to a prokaryotic or eukaryotic cell that can or has received recombinant vectors bearing one or more PKS genes, or a complete PKS cluster, and/or a polyketide modifying gene. The term includes progeny of the host cell.

[0057] An 'aglycone,' as used herein, refers to the product of a PKS enzyme that has not been modified by the addition of a sugar moiety and/or alteration by a P450 monooxygenase.

[0058] A 'control sequence' is a sequence operably linked to a gene that is capable of effecting the expression of the gene. The 'control sequence' need not be contiguous with the gene, so long as it functions to direct the expression of the gene.

[0059] As used herein, 'operably linked,' 'operatively linked' or 'operationally associated' (used interchangeably) refer to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. To optimize expression and/or *in vitro* transcription, it may be helpful to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak, *J. Biol. Chem.*, *266*:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined using techniques known in the art.

[0060] A 'megosamine biosynthetic gene set' is a gene or set of genes that confers to a heterologous host that does not produce megosamine, the ability to synthesize megosamine and, optionally, to transfer it to an aglycone. Non-limiting examples of genes belonging to a megosamine biosynthetic gene set include *megDI, megDII, megDIII, megDIV, megDV, megDVI, megDVII*, and *megBVI*.

[0061] A 'desosamine biosynthetic gene set' is a gene or set of genes that confers to a heterologous host that does not produce desosamine, the ability to synthesize desosamine and,

optionally, to transfer it to an aglycone. Non-limiting examples of genes belonging to a desosamine biosynthetic gene set include *megCII*, *megCIV*, *megCV*, *megCIII*, *megDII*, and *megDIII*.

[0062] A 'mycarose biosynthetic gene set' is a gene or set of genes that confers to a heterologous host that does not produce mycarose, the ability to synthesize mycarose and, optionally to transfer it to the appropriate attachment point on an aglycone. Non-limiting examples of genes belonging to a mycarose biosynthetic gene set include *megBI I (megBII-2)*, *megBIII*, *megBIV*, *megBV*, and *megBVI*, and *meg DIV*.

[0063] A 'modifying gene analog' is a first gene that is derived from a different organism from a second gene that performs the same function as the second gene. For example, the megK gene of the present invention derived from *M. megalomicea*, sp. nigra, the product of which hydroxylates the C-12 position of the aglycone, has a modifying gene analog *eryK* derived from *S. erythraea*.

[0064] The present invention may be practiced with reference to this disclosure and conventional methods of molecular biology and recombinant DNA techniques within the skill of one of ordinary skill in the art. Such techniques are explained in the literature, see e.g. *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987, including supplements through 2001); *Molecular Cloning: A Laboratory Manual*, third edition (Sambrook and Russel, 2001); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1999, including supplements through 2001).

(3) Description

[0065] The invention provides nucleic acids that contain polyketide modifying genes. The invention also provides vectors and host cells containing the nucleic acids, methods of using the host cells to produce glycosylated polyketides, and the glycosylated polyketides so produced.

[0066] Nucleic acids: A total genomic DNA library of *Micromonospora megalomicea*, sp. nigra, was made and cloned into cosmids, essentially as previously reported (Volchegursky, *et al.*, 2000) A series of four overlapping inserts containing the *meg* cluster were isolated from the cosmid library prepared from total genomic DNA of *M. megalomicea* that covered > 100 kb of the genome. A contiguous 48 kb segment that encodes the megalomicin PKS and several deoxysugar biosynthetic genes was sequenced and analyzed (see Fig. 1). The sequence data for the genes contained in this 48kb segment has been submitted to the DDBJ/EMBL/GenBank

database under the accession number AF263245, incorporated herein by reference. The four cosmids containing the overlapping inserts were designated pKOS079-138B, pKOS079-93A, pKOS079-93D, and pKOS205.57-2.3B. Cosmid pKOS079-93A was deposited with the American Type Culture Collection (ATCC, 10801 University Blvd., Manassas, VA), on Oct. 3, 2002 in accordance with the terms of the Budapest Treaty and is available under accession number PTA- 2555. Cosmids pKOS079-138B and pKOS205.57-2.3B were deposited with the ATCC on May 20, 2003 in accordance with the terms of the Budapest Treaty and are available under accession numbers PTA-5210 and PTA-5211, respectively. The sequences of the inserts of cosmids p pKOS079-138B and pKOS205.57-2.3B are given as SEQ ID NO: 1 and SEQ ID NO: 2, respectively. SEQ ID NO: 1 differs from a preliminary sequence of the upstream megalomicin modification genes ("preliminary sequence 1") in that preliminary sequence 1 contained a cytosine rather than an adenosine at position 59,and a cytosine rather than a thymidine at position 171, and nucleotides 5797-5799 (GGA) of SEQ ID NO:1 were deleted from preliminary sequence 1. References herein to a nucleic acid comprising SEQ ID NO:1 or portions thereof are also intended to refer to preliminary sequence 1. References herein to genes and/or ORFs that are described in terms of SEQ ID NO:1 are also intended to refer to the corresponding genes and/or ORFs of preliminary sequence 1, taking into account the above nucleotide substitutions and deletion.

[0067] The ORFs *meg*AI, *meg*AII, and *meg*AIII encode the polyketide synthase responsible for synthesis of 6-dEB. The enzyme complex *meg* DEBS is similar to *ery* DEBS, with each of the three predicted polypeptides sharing an average of 83% overall similarity with its *ery* PKS gene analog. Both PKSs are composed of six modules (two extender modules per polypeptide) and each module is organized in an identical manner. The megosamine biosynthetic genes are clustered upstream of the meg DEBS genes, while sugar modifying genes are clustered in the downstream region.

[0068] The boundaries of the ORFs of the genes of the present invention are listed in Table 1 below.

Table 1 – Open Reading Frame Boundaries

Open Reading Frame	Codon Boundaries
SEQ ID NO.1 (upstream)	

megR	52-942
megK	1051-2244
megCV	Complement 2386-3855
megCIV	Complement 3893-5098
megBVI	Complement 5095-6558
megDVI	7342-8475
megDI	8486-9024
SEQ ID NO.2 (downstream)	
megAIII (partial)	1-6965
megCII	6962-8038
megCIII	8049-9317
megBII-2	9314-10285
megH	Complement 10354-11097
megF	Complement 11105-12316
megBIII	Complement 12316-13548
megM	Complement 13928-14911
megL	Complement 14908-15972
ORF1	Complement 16326-17463

[0069] The nucleic acids of the invention may be provided in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature), purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature), and/or recombinant (i.e., nucleic acid synthesized or otherwise manipulated *in vitro*) form. Portions of nucleic acids of the invention (e.g., DNA molecules) that encode polyketide modifying enzymes (as distinguished from, e.g., vector sequences) may, in some embodiments, be fewer than about 15, 12, 10, 9, 8, 7, 6, or 5 kilobases in length. In one embodiment the portion of the nucleic acid is fewer than about 9 kilobases in length. The DNA molecules of the invention may in some embodiments also comprise one or more sequences that, in addition to polyketide modifying genes, encode one or more domains of a polyketide synthase, which may be a naturally-occurring or modified polyketide synthase. For example, the DNA molecules of the invention may in some

embodiments encode one or more domains (or fragments of such domains) of one or more modules in one or more of the ORFs of the megalomicin or other PKS. Examples of PKS domains include the KS (beta-ketoacylsynthase), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), enoylreductase (ER), acyl carrier protein (ACP), and thioesterase (TE) of at least 6 extender modules and loading module of the three proteins encoded by the three ORFs of the megalomicin PKS gene cluster.

[0070] In one aspect, a nucleic acid sequence of the invention that encodes a polyketide modifying enzyme (e.g., MegR, MegF, MegK, MegCIV, MegCV, MegBVI, MegBIII, MegL, and MegM proteins) hybridizes under stringent conditions to SEQ ID NO: 1 or 2. Typically, the nucleic acid sequence possesses at least about 90% sequence identity with a portion of SEQ ID NO: 1 or 2 that encodes a polyketide modifying enzyme. In one aspect the polyketide modifying enzyme is encoded by SEQ ID NO: 1 or 2 or a sequence that differs from the enzyme-encoding region of SEQ ID NO: 1 or 2 due to the degeneracy of the genetic code. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the nucleic acid sequences that encode them; the nucleic acid sequences and amino acid sequences encoded by the nucleic acid sequences shown herein merely illustrate preferred embodiments of the invention. The activities for the polyketide modifying enzymes are described herein.

[0071] In relation to polynucleotides and polypeptides, the term substantially identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, more preferably at least 93%, more preferably at least 95% identity, more preferably at least 96% identity, sometimes at least 97% identity or even at least about 98% identity. To determine identity, optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, 1981, *Adv. Appl. Math.* 2:482, by the search for similarity method of Pearson & Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85:2444, using the CLUSTAL W algorithm of Thompson et al., 1994, *Nucleic Acids Res* 22:467380, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI. The BLAST algorithm (Altschul et al., 1990, *Mol. Biol.* 215:403-10) for which

software may be obtained through the National Center for Biotechnology Information, see BLAST (a service of the National Center for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894) [online] program selection revised April 25, 2002 [retrieved on June 26, 2003]. Retrieved from the Internet: <URL:http://www.ncbi.nlm.nih.gov/BLAST/> can also be used. When using any of the aforementioned algorithms, the default parameters for "Window" length, gap penalty, etc., are used.

[0072] As used herein: stringency of hybridization is as follows: (1) high stringency: 0.1 x SSPE (180 mM NaCl and 10 mM NaH_2PO_4, pH 8.3), 0.1% SDS, 65°C; (2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C; and (3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C. Equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Homologs (*e.g.*, nucleic acids of the above-listed genes of species other than *Micromonospora megalomicea*) or other related sequences (*e.g.*, paralogs) can be obtained by, for example, low, moderate or high stringency hybridization with all or a portion of the particular sequence provided as a probe using methods well known in the art for nucleic acid hybridization and cloning.

[0073] The invention provides isolated, purified, or recombinant nucleic acids that contain at least one polyketide modifying gene, where the gene encodes a polyketide modifying enzyme. In some embodiments, the polyketide modifying enzyme encoded by the gene is MegR, MegF, MegK, MegCIV, MegCV, MegBVI, MegBIII, MegL, or MegM. In some embodiments, the polyketide modifying enzyme is MegR, MegK, MegCIV, MegCV, or MegBVI. In some embodiments, the polyketide modifying enzyme is MegF, MegBIII, MegL, or MegM. The gene may be operably linked to a promoter, which in some cases is a heterologous promoter. In some embodiments, the nucleic acid does not contain one or more of *megBI, megBV, megBIV, megCI, megCII, megDII, megDIII, megDIV, megDV, megDVII,* or *megY.* In some embodiment, the polyketide modifying gene encodes an amino acid sequence that is encoded by a portion of SEQ ID NO: 1 or SEQ ID NO: 2.

[0074] The invention also provides an isolated, purified, or recombinant polyketide modifying enzyme gene *meg*K, *meg*CV, *meg*CIV, *meg*R, *meg*BVI, *meg*F, *meg*BIII, *meg*L, or *meg*M.

[0075] Vectors: The nucleic acids of the invention may be inserted into a vector containing additional sequences that assist in cloning, amplification and splicing of nucleotide

sequences, and/or sequences that facilitate introduction into the cell and/or determine the relative stability and final location of the introduced nucleic acid (i.e., integrated or episomal). As used herein, the term "vector" refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors," which are designed for isolation, propagation and replication of inserted nucleotides, which may be useful for, e.g., isolating and sequencing areas of a genome of interest. An illustrative example is a cosmid vector. Vectors may also be "expression vectors," which are designed for expression of a nucleotide sequence in a host cell. Generally, the expression vector further comprises an origin of replication or a segment of DNA that enables chromosomal integration. Expression vectors may further comprise termination sequences, polyadenylation sequences, and the like, as are well-known in the art. Generally vectors are suitable for introduction into prokaryotic cells, or introduction into eukaryotic cells. Shuttle vectors are used for introduction into both eukaryotic and prokaryotic cells.

[0076] A vector used in the invention may be any vector that is compatible with the cell into which it is introduced. Conventional recombinant DNA and RNA techniques, such as those described in Sambrook, *supra*, may be used to construct vectors containing inserts that contain nucleic acids of the invention.

[0077] In some embodiments, the invention provides a cosmid vector that is pKOS079-138B or pKOS205.57-2.3B. In some embodiments, the cosmid vector contains one or more genes having a sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2; in some embodiments, the vector contains one or more genes having a sequence that is substantially identical (e.g., possessing at least 70%, 80%, 90%, 93%, 95%, 96%, 97%, or 98% identity) to SEQ ID NO: 1 or SEQ ID NO: 2; in some embodiments, the vector contains one or more genes having a sequence that hybridizes to SEQ ID NO: 1 or SEQ ID NO: 2 under stringent conditions.

[0078] The invention also provides expression vectors that contain at least one of the polyketide modifying genes described above, where the gene is operably linked to a promoter. In one embodiment, the invention provides a recombinant expression vector that comprises the desosamine biosynthetic genes, and optionally a desosaminyl transferase gene. In one embodiment, the invention provides one or more recombinant expression vectors that comprise the desosamine and mycarose biosynthetic genes, and optionally desosaminyl and mycarosyl transferase genes. In one embodiment, the invention provides one or more recombinant

expression vectors that comprise the desosamine, megosamine, and mycarose biosynthetic genes, and, optionally, desosaminyl, and mycarosyl transferase genes. In some embodiments, the polyketide modifying gene is *megR*, *megF*, *megK*, *megCIV*, *megCV*, *megBVI*, *megBIII*, *megL*, or *megM*.

[0079] Host cells: The invention further provides host cells that contain the vectors and nucleic acids of the invention. Any means, physical or biological, may be used in the methods of the present invention to introduce the nucleic acids (usually as part of a larger vector) into a cell. Means of *in vitro* introduction of foreign nucleic acid into a cell are well-known in the art, and include standard methods of transformation, transfection, and the like, including calcium phosphate precipitation, electroporation, lipofection, direct injection, DEAE-dextran, and the like (see, for example, Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990) Stockton Press, New York, NY).

[0080] The host cells of the present invention may be producers of 6-deoxysugars or may be host cells that do not naturally contain PKS genes or PKS modifying genes. The host cells of the present invention may also be natural producers of polyketides having genes for the synthesis and transfer of some deoxy sugars, for example, mycarose, but not desosamine or megosamine. In this latter case, the genes of the present invention, when introduced into said host cell confer upon the host cell the ability to synthesize one or more of the deoxysugars it lacks, for example desosamine or megosamine. Exemplary host cells of the invention include *Streptomyces coelicolor, Streptomyces lividans,* and *Micromonospora megalomicea.*

[0081] The invention provides host cells, e.g., *Streptomyces coelicolor* or *Streptomyces lividans*, that express the products the MegF and/or MegK hydroxylase genes, the megosamine biosynthesis and transfer genes of the present invention, the desosamine biosynthesis and transfer genes of the present invention, the mycarose biosynthesis and transfer genes of the present invention, and/or MegM and MegL. Thus, in some embodiments, the host cell expresses a P450-type monooxygenase enzyme, which in some cases is heterologous, and which in some cases is MegK or MegF. In some embodiments, the host cell expresses a gene from a desosamine biosynthetic gene set, where the gene is *megCIV, megCV,* or *megCIII*; in some embodiments, the gene is *megCII*, *megCIV*, *megCV*, or *megCIII*. In some embodiments, the host cell expresses a gene from a megosamine biosynthetic gene set, where the gene is *megBVI* or *megDI*; in some embodiments the gene is *megDI, megDII, megDIII, megDIV, megDV, megDVI,*

megDVII, or *megBVI*. In some embodiments, the host cell expresses a gene from a mycarose biosynthetic gene set, where the gene is *megBIII* or *megBVI*; in some embodiments, the gene is *megBII (meg BII-2)*, *megBIII*, *megBIV*, *megBV*, or *megBVI*. In some embodiments, the host cell contains an isolated, purified, or recombinant nucleic acid that encodes a polyketide modifying enzyme MegR, MegF, MegK, MegCIV, MegCV, MegBVI, MegBIII, MegL, or MegM enzymes, and expresses one or more of these enzymes. In some embodiments, the host cell contains an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of mycarose to a polyketide and/or hydroxylation of the polyketide, where the genes include the genes that encode the enzymes MegM, MegL, MegBIII, MegBIV, MegDIV, MEG BII (MegBII-2), Meg BVI, optionally MegBV, and, optionally, MegF, and expresses one or more of these enzymes. In some embodiments, the host cell contains an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of megosamine to a polyketide, where the genes may include the genes that encode the enzymes MegM, MegL, MegCII, MegBVI, MegDIV, MegDV, MegDII, and MegDIII enzymes, and, optionally the MegDI enzyme, and expresses one or more of these enzymes. In some embodiments, the host cell contains an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of megosamine to a polyketide, where the genes may include the genes that encode the enzymes MegM, MegL, MegCII, MegBVI, MegDIV, MegDVI, MegDVII, MegDII, and MegDIII enzymes, and, optionally the MegDI enzyme, and expresses one or more of these enzymes. In some embodiments, the host cell contains an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of desosamine to a polyketide, where the genes include the genes that encode the enzymes MegM, MegL, MegCII, MegCIV, MegCV, MegDII, and MegDIII enzymes, and, optionally, the MegCIII enzyme, and expresses one or more of these enzymes.

[0082] Illustrative host cells of the present invention include *Streptomyces coelicolor* and *Streptomyces lividans* cells into which the vectors of the present invention have been introduced. The invention provides, for example, an *S. coelicolor* host cell, transformed to produce the MegF and MegK hydroxylases, the mycarose biosynthesis and transfer genes of the present invention, and/or the desosamine biosynthesis and transfer genes of a different species, e.g., *S. erythraea*. These host cells illustrate how one can use certain recombinant genes of the present invention with modifying gene analogs to create host cells of the invention.

[0083] Another illustrative host cell of the present invention is an *E. coli* host cell transformed with vectors having the *megAI, megAII, and megAIII* PKS genes to make 6-dEB; the genes for MegM glucose-6-dehydrogenase and MegL TDP-glucose-synthase to make deoxysugars; the genes for MegF and MegK P450-type monooxygenases to hydroxylate the 6-dEB aglycone at the C-6 and C-12 positions respectively; the mycarose biosynthesis and transferase genes; and the desosamine biosynthesis and transferase genes. In another embodiment, the host cell further comprises the megosamine biosynthesis and transferase genes.

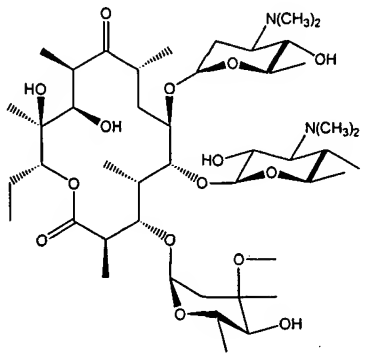
[0084] Methods and compounds: The invention also provides methods for producing hydroxylated and glycosylated polyketides using the nucleic acids, vectors, and host cells described herein, by culturing a host cell that contains an expression vector of the invention under conditions where the cell produces a polyketide that is then modified. The cell may be unable to make the polyketide in the absence of the expression vector. For example, in some embodiments, the cell in its natural, non-recombinant state is unable to produce 6dEB. Methods of culturing host cells, such as those provided by the invention, to produce a polyketide are known in the art.

[0085] In an illustrative embodiment, the polyketide is a derivative of 6-dEB that has a group other than an ethyl moiety at C-13 (13-R-6-dEB, where R is not ethyl). Methods for making 13-R-6-dEB compounds in an *S. coelicolor* host cell, which lacks genes for polyketide modification enzymes, are described in U.S. Patent Nos. 6,080,555; 6,274,560; 6,066,271; and 6,261,816, as well as PCT Pub. Nos. 98/49315; 99/03986; and 00/44717. These 13-R-6-dEB compounds can be converted to the corresponding 13-R-erythromycins by feeding the aglycones to a fermentation of *S. erythraea*, as described in the aforementioned patent publications. The 13-R-erythromycins can be converted chemically into potent antibiotics known as ketolides, as described in PCT Pub. Nos. 00/63225; 00/62873; and 00/63224, each of which is incorporated herein by reference. The present invention provides methods and reagents for making the 13-R-erythromycins in a single fermentation, as opposed to two fermentations, in that the invention provides a host cell that contains the requisite hydroxylase genes and desosamine and mycarose biosynthesis and transferase genes from the megalomicin biosynthetic gene cluster as well as the PKS for making the 13-R-erythromycins. The PKS genes and the corresponding mutated versions (which contain the KS1 null mutation) that produce a PKS that can convert a diketide into a 13-R-6-dEB can be obtained as described in PCT Pub. No. 01/27284 (the *meg* PKS genes);

U.S. Patent No. 6,251,636 (the *ole* PKS genes); and U.S. Patent No. 6,080,555 (the *ery* PKS genes), each of which is incorporated herein by reference. This host cell of the invention produces 13-R-erythromycin C compounds, instead of 13-R-erythromycin A compounds, because the host cell lacks the *eryG* gene that converts the mycarosyl residue to a cladinosyl residue. In other embodiments, the host cell is provided with a recombinant *eryG* gene and makes the corresponding 13-R-erythromycin A derivatives. In another embodiment, the host cell contains PKS genes that do not comprise the KS1 null mutation and so produce erythromycins A, B, C, and/or D. Thus, the host cells of the invention can be used to produce erythromycin and erythromycin analogs that can be converted to ketolides.

[0086] In one embodiment, the invention provides *Streptomyces lividans* and *Streptomyces coelicolor* host cells transformed with a vector or vectors including the PKS genes (*megAI*, *megAII*, and *megAIII*), and the genes for hydroxylation and for production and transfer of glycosyl units, as shown in Figs. 2 and 3: mycarose genes (*eryG, megL, megM, megDIV,* and all *megB* genes), desosamine genes (*megL, megM, meg DII* and *meg DIII, and* all *megC* genes), megosamine genes (*megL, megM, meg BVI,* and all *megD* genes), and *megK* and *megF* genes and the transformed host is cultured under conditions that lead to the production of polyketides resulted in the production of novel biologically active compounds, such as the compound of formula (1) having a methyl group in the 3''' position of the mycarose sugar moiety of megalomicin. This compound is believed to be a more potent antibiotic against certain pathogens than megalomicin.

6-O-Megosaminylerythromycin A
3'''-O-Methylmegalomicin A



Formula 1

[0087] In another embodiment, the invention provides a method for making a polyketide of formula (1) as follows. A vector including a functional *eryG* gene and a disrupted *megG* (previously designated *megY*) gene is transformed into an *M. megalomicea* host and the transformed host cultured under conditions such that polyketides are produced. This results in the production of the compound of formula (1) having a methyl group in the 3''' position of the mycarose sugar moiety of megalomicin.

[0088] The invention also provides a method of producing the polyketide of Formula (1) by culturing a cell that expresses one or more polypeptides encoded by a recombinant polynucleotide that includes the genes *megDII, DIII, DIV, DV, DVI,* and *DVII*, and optionally includes extra copies of an *eryG* gene, and does not include a *megY* gene, where the cell produces erythromycin A in the absence of the recombinant polynucleotide, under conditions where the cell produces the polyketide.

[0089] The invention further provides a method of producing the polyketide of Formula (1) by culturing a cell that is a *Streptomyces coelicolor* or a *S. lividans* cell, where the cell expresses one or more polypeptides encoded by a recombinant polynucleotide that includes the genes *megAI, megAII,* and *megAIII*; mycarose genes that include all *megB* genes and the *megDIV* gene; desosamine genes that include all *megC* genes and the *megDII* and *megDIII* genes; megosamine genes that include all *megD* genes; *eryG, megL, megM* and *megK* and *megF*; under conditions where the cell produces the polyketide.

[0090] The invention further provides a method of producing the polyketide of Formula (1) by culturing a *Micromonospora megalomicea* cell that contains a recombinant polynucleotide that includes an *eryG* gene under control of a regulator or promoter, where the *megY* gene of the host cell is disrupted or its product is inactivated, under conditions where the cell produces the polyketide.

[0091] The invention further provides a method for producing 3-O-α-mycarosyl-erythronolide B in heterologous host (see, e.g., Example 7) by introducing an isolated, purified, or recombinant nucleic acid containing genes for the biosynthesis and attachment of mycarose to a polyketide, where the genes include the genes that encode the enzymes MegM, MegL, MegBIII, MegBIV, MegDIV, Meg BV, Meg BII (MegBII-2), Meg BVI, and, optionally, MegF, into a heterologous host cell, e.g., *S. coelicolor*, and culturing the cells under conditions where

the 3-O-α-Mycarosyl-Erythronolide B is produced. Such conditions in *S. coelicolor* are, for example, YEME medium with thiostreptin, fed with 6-deoxyerythronolide B (see Example 7).

[0092] The invention further provides the polyketide of Formula (1). In some embodiments, the polyketide is isolated and/or purified. Methods for isolation and purification of polyketides are known in the art.

[0093] Thus the recombinant genes of the invention, and the portions thereof, are useful for a variety of purposes, including production of novel megalomicin analogs. BLAST (Altschul *et al.,* 1990) analysis of the genes flanking the meg PKS genes indicates that 13 complete open reading frame (ORFs) appear to encode functions required for synthesis of at least one of the three megalomicin deoxysugars. Each ORF was assigned to a specific deoxysugar pathway based on comparison to PKS genes and other related genes involved in deoxysugar biosynthesis. Three ORFs, *megBV, megCIII* and *megDI,* encode glycosyl-transferases, one for attachment of each different deoxysugar to the macrolide. MegBV was assigned to the mycarose pathway in the *meg* cluster. In similar fashion, assignments were made accordingly for: MegCII and MegDVI, two 3,4-isomerases homologous to EryCII; MegBII (MegBII-2) and MegDVII (MegBII-1), 2,3-reductases homologous to EryBII; MegBIV and MegDV, putative 4-ketoreductases similar to EryBIV. The remaining ORFs involved in deoxysugar biosynthesis, *meg*BVI (also known as *meg*T), *meg*DII, *meg*DIII and *meg*DIV, each encode a 2,3-dehydratase, aminotransferase, dimethyltransferase and 3,5-epimerase respectively. As both the megosamine and desosamine pathways require an aminotransferase and a dimethyltransferase, and as mycarose and megosamine each require a 2,3-dehydratase and a 3,5-epimerase, assignments of these four genes to a specific pathway could not be made on the basis of sequence comparison alone.

[0094] Additional complete ORFs *megG* (also designated *megY*), *megH*, *megK* and *megF* were also identified in the cluster with sequence to the encoded proteins MegH, MegK, and MegF. The proteins MegH, MegK and MegF share high degrees of similarity with EryH, EryF, and EryK respectively. EryH and homologues in other macrolide gene clusters are thioesterase-like proteins (Haydock *et al.,* 1991; Xue *et al.,* 1998; Butler *et al.,* 1999; Tang *et al.,* 1999). This gene can be inserted in a heterologous host or disrupted in the native host to increase production of a desired polyketide. The *eryF* gene encodes the erythronolide B C-6 hydroxylase (Fig. 2) (Weber *et al.,* 1991; Andersen and Hutchinson, 1992). The *eryK* gene encodes erythromycin D

C-12 hydroxylase. The *megY* gene does not have an *ery* counterpart, but is believed to belong to a (small) family of O-acyltransferases that transfer short acyl chains to macrolides (Hara, O., *et al.* 1992). The structures of various megalomicins places *megY* in the latter class as the acyltransferase that converts megalomicin A to megalomicins B, C1 or C2.

[0095] An examination of the *meg* cluster reveals that the megosamine biosynthetic genes are clustered directly upstream of the PKS genes. The hypothesis that these genes are sufficient for biosynthesis and attachment of megosamine to a macrolide intermediate was confirmed by functional expression of these genes in a strain which produces erythromycin, such as *S. erythraea,* resulting in production of megalomicin (See Example 3). Expression of *megDVI-megDVII* segment in *S. erythraea* and the corresponding production of megalomicins in this host established the likely order of sugar attachment in megalomicin synthesis (See Fig.2). Furthermore, it has provided a means to produce megalomicin in a more genetically friendly host organism, leading to the creation of megalomicin analogues by manipulating the megalomicin PKS.

[0096] Because introduction of this *meg* DNA segment into *S. erythraea* results in production of megalomicins, it is clear that these genes encode the functions for TDP-megosamine biosynthesis and transfer to its substrate and to acylate the polyketide (see Fig. 2). The remaining region upstream of *megDVI* includes genes for mycarose and desosamine biosynthesis. Furthermore, if the organization resembles that of the left arm of the *ery* cluster, the megosamine biosynthesis 'island' may have been formed via an insertion of the *megD* and *megY* genes into an existing erythromycin or other common ancestral gene cluster.

[0097] The entire gene set from *megDI* to *meg*DVII was introduced in *S. erythraea* to produce TDP-megosamine. Two alternative pathways are possible. One pathway converts TDP-2,6-dideoxy-3,4-diketo-hexose (or its enol tautomer), the last intermediate common to the mycarose and megosamine pathways, to TDP-megosamine through the sequence of 5-epimerization, 4-ketoreduction, 3-amination and 3-N-dimethylation using the genes *meg*DIV, *meg*DV, *meg*DII *and meg*DIII (Fig. 3). This pathway uses the same functions proposed for biosynthesis of TDP-daunosamine by Olano *et al.* (1999) but in a different sequential order. However, it does not account for the *meg*DVI and *meg*DVII genes as their encoded activities are not required in this pathway. A parallel pathway that uses these genes is also shown in Fig. 3. In this alternative route, 2,3-reduction and 3,4-tautomerization are performed by the *meg*DVII and

*meg*DVI gene products, respectively. To confirm which alternative pathway is utilized in a host cell, gene disruption and complementation experiments can be conducted.

[0098] The 48 kb segment sequenced also contains genes required for synthesis of TDP-L-mycarose and TDP-D-desosamine (Fig. 3). The *meg*CII gene encodes a putative 3,4-isomerase which catalyses the presumed first step in the committed TDP-desosamine pathway. The start codon of *meg*CII overlaps the stop codon of *meg*AIII in exactly the same manner as their erythromycin counterparts *ery*CII and *ery*AIII overlap (Summers *et al.,* 1997), suggesting that these genes are translationally coupled in both systems. The high degree of similarity between MegCII and EryCII indicates that the pathway to desosamine in the megalomicin-producing and erythromycin-producing organisms is similar. Similarly, the finding that *megBII (megBII-2)* and *megBIV,* encoding a 2,3-reductase and 4-ketoreductase, contain close homologues in the mycarose pathway for erythromycin also suggests that TDP-L-mycarose synthesis in the two host organisms is the similar.

[0099] Of note are the two genes that encode putative 2,3-reductases *meg*BII (*meg BII-2*) and *meg*DVII (*megBII-1*). Because MegBII (MegBII-2) most closely resembles EryBII, a known mycarose biosynthetic enzyme (Weber *et al.*, 1990), and because *meg*BII resides in the same location of the *meg* cluster as its counterpart in the *ery* cluster, *meg*BII (*megBII-2*) was assigned to the mycarose pathway and *meg*DVII (*megBII-1*) to the megosamine pathway. Furthermore, the lower degree of similarity between MegDVII (*megBII-1*) and either EryBII or MegBII (*megBII-2*) (Table 1) provided a basis for assigning the opposite L- and D-isomeric substrates to each of the enzymes (Fig. 3). Finally, *meg*BVI, which encodes a putative 2,3-dehydratase, is also related to *ery*BVI gene in the *ery* mycarose pathway. In *S. erythraea,* the proposed intermediate generated by EryBVI represents the first committed step in the biosynthesis of mycarose (Fig. 3). However, the proposed pathways in Fig. 3 suggest that this may be an intermediate common to both mycarose and megosamine biosynthesis in *M. megalomicea.*

[0100] The recombinant genes, vectors, and host cells of the invention have a wide variety of useful applications. Host-vector systems for expression of *meg* DEBS genes and other heterologous expression of modular PKS genes for erythromycin (Kao *et al.*, 1994b; Ziermann and Betlach, 1999), picromycin (Tang *et al.,* 1999) and oleandomycin (Shah *et al.,* 2000) as well as for the generation of novel polyketide backbones in which domains have been removed, added or exchanged in various combinations (McDaniel *et al.,* 1999) have been described. Hybrid

polyketides have been generated through the co-expression of subunits from different PKS systems (Tang *et al.,* 2000). The present invention provides materials and methods of producing modified polyketides in heterologous hosts by the addition, replacement, or removal of modifying sugar moieties and/or hydroxyl groups on the polyketide core.

[0101] A detailed description of the invention having been provided, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

EXAMPLE 1

Materials and Methods

[0102] Strains. Routine DNA manipulations were performed in *Escherichia coli* XL1 Blue or *E. coil* XLI Blue MR (Stratagene) using standard culture conditions (Sambrook *et al.,* 1989). *M. megalomicea* subs. *nigra* NRRL3275 was obtained from the ATCC collection and cultured according to recommended protocols. For isolation of genomic DNA, *M. megalomicea* was grown in tryptone soya broth (TSB) (Hopwood *et al.,* 1985) at 3000 rpm. *S. lividans* K4-114 (Ziermann and Betlach, 1999), which carries a deletion of the actinorhodin biosynthetic gene cluster, was used as the host for expression of the *meg* DEBS genes (see U.S. Patent No. 6,177,262). *S. lividans* strains were maintained on R5 agar at 30^0C and were grown in liquid yeast extract—malt extract (YEME) for preparation of protoplasts (Hopwood *et al.,* 1985). *S. erythraea* NRRL2338 was used for expression of the megosamine genes. *S. erythraea* strains were maintained on R5 agar at 34°C and grown in liquid TSB for preparation of protoplasts.

[0103] (B) Manipulation of DNA and Organisms. Manipulation and transformation of DNA in *E. coli* was performed according to standard procedures (Sambrook *et al.,* 1989) or to suppliers' protocols. Protoplasts of *S. lividans* and *S. erythraea* were generated for transformation by plasmid DNA using the standard procedure (Hopwood *et al.,* 1985). *S. lividans* transformants were selected on R5 using 2 ml of a 0.5 mg/ml thiostrepton overlay. *S. erythraea* transformants were selected on R5 using 1.5 ml of a 0.6 mg/ml apramycin overlay.

[0104] (C) DNA Sequencing and Analysis. PCR-based double-stranded DNA sequencing was performed on a Beckman CEQ 2000 capillary sequencer using reagents and protocols provided by the manufacturer. A shotgun library of the entire cosmid pKOSO79-93D insert was made as follows: DNA was first digested with *DraI* to eliminate the vector fragment, then partially digested with Sau3AI. After agarose electrophoresis, bands between 1 and 3 kb were

excised from the gel and ligated with *BamHI* digested pUC19. Another shotgun library was generated from a 12 kb *XhoI—EcoRI* fragment subcloned from cosmid pKOSO79-93A to extend the sequence to the *megF* gene. A 4 kb *BglII—XhoI* fragment from cosmid pKOS079-138B was sequenced by primer walking to extend the sequencing to the *megBVI* gene. Sequence was assembled using the SEQUENCHER (Gene Codes) software package and analysed with MacVector (Oxford Molecular Group) and the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/blast/).

EXAMPLE 2

Isolation of the Megalomicin Biosynthetic Gene Cluster

[0105] A cosmid library was prepared in SuperCos (Stratagene) vectors from *M. megalomicea* total DNA partially digested with Sau3AI and introduced into *E. coli* using a Gigapack III XL (Stratagene) *in vitro* packaging kit. ^{32}P-labelled DNA probes encompassing the KS2 domain from DEBS, or a mixture of segments encompassing modules 1 and 2 from DEBS, were used separately to screen the cosmid library by colony hybridization. Several colonies which hybridized with the probes were further analyzed by sequencing the ends of their cosmid inserts using T3 and T7 primers. BLAST (Altschul *et al.,* 1990) analysis of the sequences revealed several colonies with DNA sequences highly homologous to genes from the *ery* cluster. Together with restriction analysis, this led to the isolation of two overlapping cosmids, pKOSO79-93A and pKOSO79-93D which covered ~ 45 kb of the *meg* cluster. A 400 bp PCR fragment was generated from the left end of pKOSO79-93D and used to reprobe the cosmid library. Likewise, a 200 bp PCR fragment generated from the right end of pKOSO79-93A was used to reprobe the cosmid library. Analysis of hybridizing colonies, as described above, resulted in identification of two additional cosmids pKOSO79-138B adjacent to the 5' end of pKOS079-93D and pKOS205.57-2.3B which overlaps the 3' ends of pKOS079-93A and pKOS079-93D cosmids. See Fig. 1.

[0106] BLAST analysis of the far left and right end sequences of these cosmids indicated no homology to any known genes related to polyketide biosynthesis, and therefore indicates that the set of four cosmids spans the entire megalomicin biosynthetic gene cluster.

[0107] The glycosyl synthase, transfer, and regulatory genes of the upstream region of the *meg* PKS are contained in the nucleotide sequence SEQ ID No. 1.

[0108] The glycosyl synthase, and transfer genes of the downstream region of the *meg* PKS are contained in the nucleotide sequence SEQ ID NO: 2.

EXAMPLE 3

Production of a Modified Polyketide in a Heterologous Host

[0109] Fermentation for production of polyketide, LC/MS analysis, and quantification of 6-dEB for *S. lividans* K4-114/pKOS1O8-6 and *S. lividans* K4-114/pKAO127 'kan' were essentially as previously described (Xue *et al.*, 1999). *S. erythraea* NRRL2338 and *S. erythraea/pKOS97-42* were grown for 6 days in F1 medium (Brünker *et al.*, 1998). Samples of broth were clarified in a microcentrifuge (5 mm, 13 000 rpm). For LC/MS preparation, isopropanol was added to the supernatant (1:2 ratio), and the supernatant centrifuged again. Samples were run on a C-18 reversed phase column (Inertsil ODS3, Metachem) using a 5-mM ammonium acetate (aqueous) acetonitrile— methanol (4:1) gradient (0—15%, 3 mm; 15—60%, 10 mm; 1 ml/min flow). Erythromycins and megalomicins were detected by electrospray mass spectrometry and quantity was determined by evaporative light scattering detection (ELSD). A purified extract from *M. megalomicea* containing megalomicin A, B, C1 and C2 was used for the standard reference. The LC retention time and mass spectra of erythromycin and the four megalomicins were identical to those from the standards. Thus the, *S. erythraea* host cell of the invention produced megalomicin in detectable and useful quantities.

EXAMPLE 4

Plasmids Incorporating Glycosyl Synthase and Transferase Genes

[0110] Plasmid pKOS108-6 is a modified version of pKAO127'kan' (Ziermann and Betlach, 1999, 2000), in which the *eryAI—III* genes between the *PacI* and *EcoRI* sites have been replaced with the *megAI—III* genes. This was carried out by first substituting a synthetic nucleotide DNA duplex (5'- TAAGAATTCGGAGATCTGGCCTCAGCTCTAGAC (SEQ ID NO: 3), complementary oligo-5'-AATTGTCTAGAGCTGAGGCCAGATCTCCGAATTCTTAAT (SEQ ID NO: 4)) between the *PacI* and EcoRI sites of the pKAO127'kan' vector fragment. The 22 kb *EcoRI—BglII* fragment from cosmid pKQS079-93D containing the *megAI—II* genes was inserted into *EcoRI* and *BglII* sites of the resulting plasmid to generate pKOS024-84. A 12 kb *BglII—BbvCI* fragment containing the *megAIII* and part of the *megCII* gene was subcloned from pKOS079-93A and

excised as a *BglII—XbaI* fragment and ligated into the corresponding sites of pKOS024-84 to yield the final expression plasmid pKOS1O8-06. The megosamine integrating vector pKOS97-42 was constructed as follows: a subclone was generated containing the 4 kb *XhoI—ScaI* fragment from pKOS79-138B together with the 1.7 kb *ScaI—PstI* fragment from pKOS79-93D in Litmus 28 (Stratagene). The entire 5.7 kb fragment was then excised as a *SpeI—PstI* fragment and combined with the 6.3 kb *PstI—EcoRI* fragment from K0S79-93D and *EcoRI— XbaI*-digested pSET152 (Bierman *et al.*, 1992) to construct plasmid pKOS97-42.

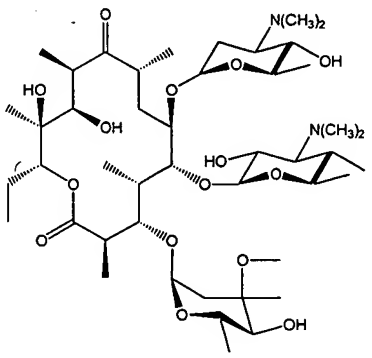
[0111] Cosmid pKOS79-138B contains the genes *meg*R, *meg*K, *meg*CV, *meg*CIV, and *meg*BVI.

[0112] Cosmid pKOS205.57-2.3B contains the genes *meg*CII, *meg*CIII, *meg*BII-2, *meg*H, *meg*F, *meg*BIII, and *meg*M and *meg*L.

EXAMPLE 5

Production of Polyketide 3'''-O-methylmegalomicin A in a Heterologous Host

6-O-Megosaminylerythromycin A
3'''-O-Methylmegalomicin A



Formula (1)

[0113] A) *Saccharopolyspora erythraea* - erythromycin A producing strain.

Fermentation for production of polyketide, LC/MS analysis and quantification of 6-dEB for *S. erythraea* are essentially as described in Example 3. Plasmid vectors comprising the *meg*D genes (DI, DII, DIII, DIV, DV, DVI, and DVII), are transformed into an erythromycin A producer strain of *Saccharopolyspora erythraea* excluding the *meg*Y gene, and optionally, extra copies of the *ery*G gene are provided. Culturing the transformed host cell under conditions that lead to the

production of the compound of formula (1) having a methyl group in the 3''' position of the mycarose sugar moiety of megalomicin.

[0114] B) *Streptomyces coelicolor, S. lividans* or other heterologous host. Fermentation for production of polyketide, LC/MS analysis, and quantification of 6-dEB for *S. lividans* and *S. coelicolor* are essentially as described in Example 3. A vector or vectors including the PKS genes (*megAI*, *megAII*, and *megAIII*), mycarose genes (all *megB* genes), desosamine genes (all *megC* genes), megosamine genes (all *megD* genes), and *megK* and *megF* genes, *eryG* gene and optionally the *megL* and *megM* genes (the *megL* and *megM* genes can be considered members of the mycarose, desosamine, or megosamine biosynthetic gene sets in host cells that lack an analog gene of either) are transformed into *S. lividans* and *S. coelicolor*, and the transformed host is cultured under conditions that lead to the production of the compound of formula (1) having a methyl group in the 3''' position of the mycarose sugar moiety of megalomicin.

[0115] C) *Micromonospora megalomicea*. Fermentation for production of polyketide, LC/MS analysis, and quantification of 6-dEB for *Micromonospora megalomicea* are essentially as described in Example 3. A vector including a functional *eryG* gene and a disrupted *megY* are transformed into an *M. megalomicea* host, and the transformed host is cultured under conditions that lead to the production of the compound of formula (1) having a methyl group in the 3''' position of the mycarose sugar moiety of megalomicin.

EXAMPLE 6

Production of Erythronolide B in a Heterologous Host

[0116] The gene encoding a cytochrome P450 monooxygenase of the megalomicin cluster, *megF*, was PCR amplified and cloned into plasmid pET21, yielding plasmid pLB73. In this plasmid, *megF* is under the control of the ϕ10 promoter of T7. Plasmid pLB73 was transformed into *E. coli* BL21 (DE3) and selected for resistance to apramycin.

Five ml of LB medium containing 100 μg/ml of ampicillin was inoculated with a fresh colony of BL21/pLB73. When the culture reached an OD_{590} of 0.6 the expression of *megF* was induced by addition of 0.5 μM of IPTG, and the culture was incubated for 20 h at 37 °C in the presence of 100 μg of 6-dEB. The culture was centrifuged, and the supernatant was extracted with 5 mL of ethyl acetate and the organic phase dried under a stream of N_2. LC/MS analysis of the sample confirmed that approximately 50 % of the 6-dEB had been converted into EB. LC

conditions were as follows: MetaChem ODS-3 5 um reversed phase column, 4.6 x 150 mm; flow rate 1 mL/min; gradient of 35% to 100% acetonitrile in water over 8 minutes; MS detection using a PE-Sciex API100LC mass sensitive detector at 1 amu resolution from 200-1200 amu with an APCI ion source.

EXAMPLE 7

Production of 3-O-α-Mycarosyl-Erythronolide B in a Heterologous Host

[0117] Genes involved in the biosynthesis of mycarose were individually amplified by PCR using Deep Vent DNA polymerase (commercially available from NEB) from *M. megalomicea* chromosomal DNA with the following primers:

megL

forward: 5′-GGGGTCATATGAAGGCGCTTGTCCTGTCGG-3′ (SEQ ID NO:5);

reverse: 5′-GCAAAGCTTGTGACTAGTCGAGTAGTC-3′ (SEQ ID NO:6);

megM

forward: 5′-GACCTCCATATGACGACTCGACTCCTGGTC-3′ (SEQ ID NO:7);

reverse: 5′-TACTAGTCCCTCACACCATCGCCCG-3′ (SEQ ID NO:8);

megBIII

forward: 5′-CAGCATATGCCCGAAACGAGATGCCG-3′ (SEQ ID NO:9);

reverse: 5′-ATCGACTAGTTTCATCACACCACTTCCAGG-3′ (SEQ ID NO:10);

megBIV

forward: 5′-GCATATGACAAGACATGTCACACTTCTCGG-3′ (SEQ ID NO:11);

reverse: 5′-CCCACTAGTGTCACTCCTTGGTCGAGATGA-3′ (SEQ ID NO:12);

megF

forward: 5′-TGGTCATATGAAACTGCCCGATCTGGAGAG-3′ (SEQ ID NO:13);

reverse: 5′-CATACTAGTCTCATCCGTTCGGTCGCACCG-3′ (SEQ ID NO:14);

megDIV

forward: 5′-CCGGGCATATGAGGGTCGAGGAGCTG-3′ (SEQ ID NO:15);

reverse: 5′-GCACACTAGTCCGGGGTCACGTCCGC-3′ (SEQ ID NO:16);

megBV

forward: 5′-TGTACATATGCGGGTCCTGCTCACCTCG-3′ (SEQ ID NO:17);

reverse: 5′-ACACTAGTCACCTGTCGGCGCGGTGCTG-3′ (SEQ ID NO:18);

megBII-2

forward: 5′-CCGTCATCTGAGCACCGACGCCAC-3′ (SEQ ID NO:19);

reverse: 5′-AGGACTAGTGCGGGCTCTCACCGTAG-3′ (SEQ ID NO:20);

megBVI

forward: 5′-GGCATATGGGGGATCGGGTCAACGGTCATG-3′ (SEQ ID NO:21);

reverse: 5′-GTACTAGTTTCACGCCGTCGCCCGGTTGTAG-3′ (SEQ ID NO:22);

[0118] Each pair of primers introduces an *Nde*I site at the 5′ end and a *Spe*I site at the 3′ end of the gene amplified. PCR products were cloned into pCR-Blunt II-TOPO vector and the resulting plasmids were used to transform *E. coli* DH5α. The plasmids were digested with the enzymes *Nde*I and *Spe*I and fragments corresponding to each gene were cloned into a modified pET-24b previously digested with the same enzymes. The modifications introduced in the vector were the following: the region between the *Xba*I and *Eco*RI sites in the MCS was replaced by the sequence

5′-TCTAGAAGGAGATATACATATGTGAACTAGTGAATTC -3′ (SEQ ID NO:23) or by the sequence 5′-

TCTAGAAGGAGATATACAATGCACCACCACCACCACCATATGTGAACTAGTGAATTC -3′ (SEQ ID NO:24) in case His-Tag fusions were required. These sequences contained the following sites *Xba*I, *Nde*I, *Spe*I and *Eco*RI restriction sites and the pET-24b RBS.

[0119] Plasmid DNA carrying the *megL* gene was digested with the enzymes *Xba*I and *Spe*I and the 1.1 kb fragment was cloned into the plasmid harboring the *megM* gene, previously digested with the enzyme *Spe*I. Clones with *megM* and *megL* genes in the same orientation were selected. The resulting plasmid was digested with the enzyme *Spe*I and was ligated to the 1.2 kb fragment obtained by digestion of the plasmid harboring *megBIII* gene with the enzymes XbaI and SpeI. Sequential cloning of the remaining genes into the pET-24b based vector was performed with the same pattern of restriction enzymes digestions and ligations. This resulted in construction of pLB80 with a 9.7 kb operon comprising nine genes involved in the biosynthesis of mycarose, in the following order: *megM-megL-megBIII-megBIV-megF-megDIV-megBV-megBII-2-megBVI.*

[0120] pLB80 plasmid was digested with the enzymes *Xba*I and *Hin*dIII and the 9.7 kb fragment was cloned into the plasmid pKOS146-83A digested with the same restriction enzymes, leaving the artificial mycarose operon under the control of the P*actIII* promoter. The

resulting plasmid was digested with the enzymes *Eco*RI and *Spe*I and the 10.3 kb fragment was cloned into the plasmid pWHM3 digested with the enzymes *Xba*I and *Eco*RI to give the plasmid pLB92. Plasmid pLB92 was used to transform the *S. coelicolor* strain M145.
Cultures of *S. coelicolor* M145 harboring the pLB92 plasmid were grown in YEME with thiostrepton (5 μg/ml) at 30°C. Cultures were fed with 6-deoxyerythronolide B (0.5 μg/ml) and after 96 hs they were centrifuged, and the supernatants were adjusted to pH 9-10 with sodium hydroxide. The supernatants were extracted with an equal volume of ethyl acetate and the organic layer was dried over Na_2SO_4 , evaporated to dryness and redisolved in ethanol. The presence of 3-O-α-mycarosyl-erythronolide B was confirmed by LC/MS.

[0121] Although the present invention has been described in detail with reference to specific embodiments, those of skill in the art will recognize that modifications and improvements are within the scope and spirit of the invention, as set forth in the claims which follow. All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an admission that any such document is pertinent prior art, nor does it constitute any admission as to the contents or date of the same. The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.

SEQ ID NO.1 – Sequence Containing Upstream Megalomicin Modification Enzyme Genes – pKOS079-138B

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   1 GCGCGCTTCG ATCACCATGG ATCGCTTAAT GTCCGGTTCC ATTGCTTTTC GATGGGGGAT
  61 GTAGTGCAAT TGCAAAATCC GGAGACCGTG GTAAGCCTCG GAGTCCTGGG TCCGCTGTTC
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SEQ ID NO.2 – Sequence Containing Downstream Megalomicin Modification Genes – KOS205-57-2.3B

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